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Large Panel Targeted Proteomics for Low-amount Samples Using an Enhanced Nanoflow 6495D LC/TQ System

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Introduction

Quantitative analysis of low amount samples, e.g., single cell proteome, requires highly sensitive, precise and accurate analytical instruments. This study demonstrated significant enhancement using a modified nanoflow LC/MS system, including Evosep One LC, Newomics ion source and Agilent 6495D Triple Quadrupole LC/MS system, for large panel targeted proteome quantification.

The key improvements were carried out on two aspects:

1) The Newomics UniESI ion source was modified to improve the usability and electrospray robustness;
2) The Agilent MassHunter acquisition software was revised to support 10K dMRMs in a single method on the 6495D LC/TQ, which greatly accelerated method development and improved protein/peptide detection accuracy in complex matrices.

These enhancements collectively increased the selectivity, sensitivity, and throughput of the MRM-based method, making it more effective for proteome measurement and protein biomarker discovery in low-amount protein samples.

Experimental

Instrumentation

- Evosep One LC
- Newomics UniESI 2.0 ion source
- Agilent 6495D triple quadrupole LC/MS system (G6495D)
- Agilent AssayMAP Bravo platform (G5571AA)

Materials

- Human K562 cell protein digest standard (Promega).
- Evotip (Evosep, Inc.)
- IonOpticks Aurora Elite XT column (15 cm x 75 µm ID, 1.7 µm C18)

Method Development

A large panel dynamic MRM method covering 1,000 human proteins (one peptide per protein group, eight product ions per precursor) was developed based on an existing human K562 peptide spectrum library without any stable isotope-labeled (SIL) peptide (Figure 1). Skyline software (v24.1.0.199) was used for targeted peptide/transition selection, method optimization and data analysis.

Experimental

LC/MS Analysis

The K562 cell proteome digest standard was serially diluted by a factor of two. Twelve levels of standard samples, ranging from 31.25pg to 64 ng on-column, were analyzed with replicates using the 1000-protein dMRM method with the whisper zoom 20 SPD LC method. The column was heated at 50°C. LC/MS/MS data acquisition was carried out using MassHunter software (v12.2 with revision).

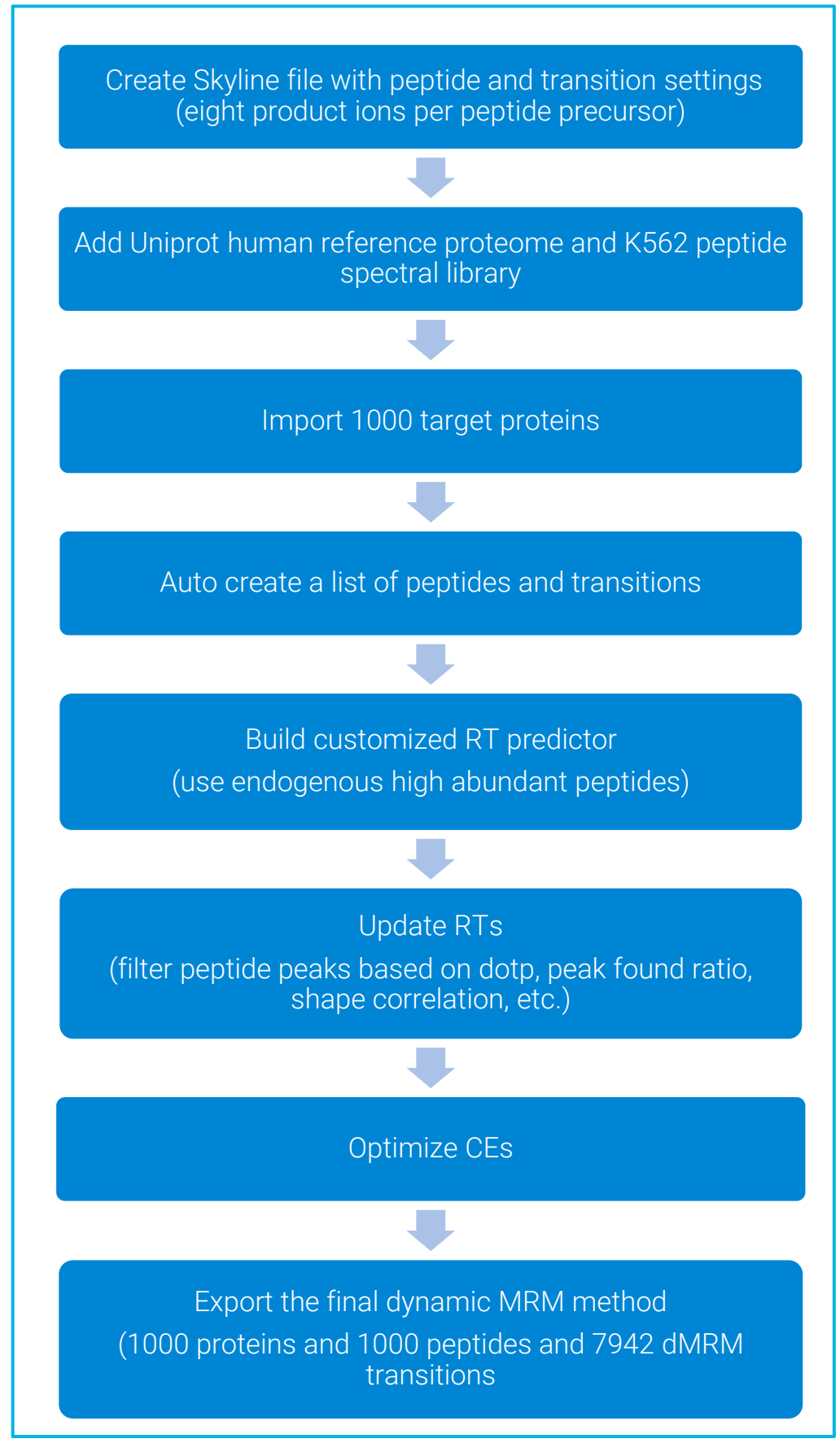

Figure 1. Dynamic MRM method development workflow with Skyline software and Agilent automation plugin using human proteome sample and peptide spectral library without SILs.

Results and Discussion

Full Study Workflow

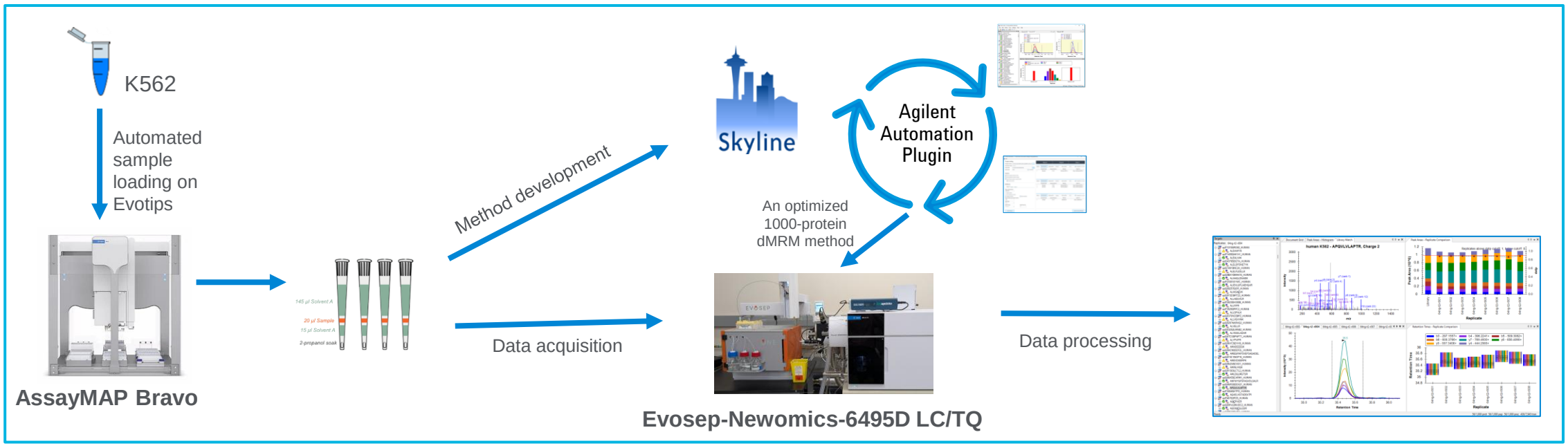

Figure 2. Diagram of study workflow for MRM-based targeted proteomics assay development, sample analysis and data processing.

Method Development

The comprehensive dMRM method was developed and optimized using Skyline software with Agilent automation plugin on the 6495D LC/TQ (Figure 1). The whole workflow was based on the endogenous K562 cell proteins without any SIL peptides. Eight transitions per peptide precursor were pre-selected based on peptide spectral library for high confidence detection.

To evaluate the detection accuracy of the MRM-based method development workflow, the same LC, analytical column and ion source was connected to a high-resolution LC/QTOF for DDA-based proteomic identification. The retention times (RTs) of 456 common peptides detected in both workflows were compared (Figure 3). 98.5% of the common peptides show consistent RTs, demonstrating high detection accuracy by the MRM-based method development workflow. Increased transition number per peptide greatly improves detection accuracy compared to a previous study (four transitions per peptide, 68% accuracy)[1].

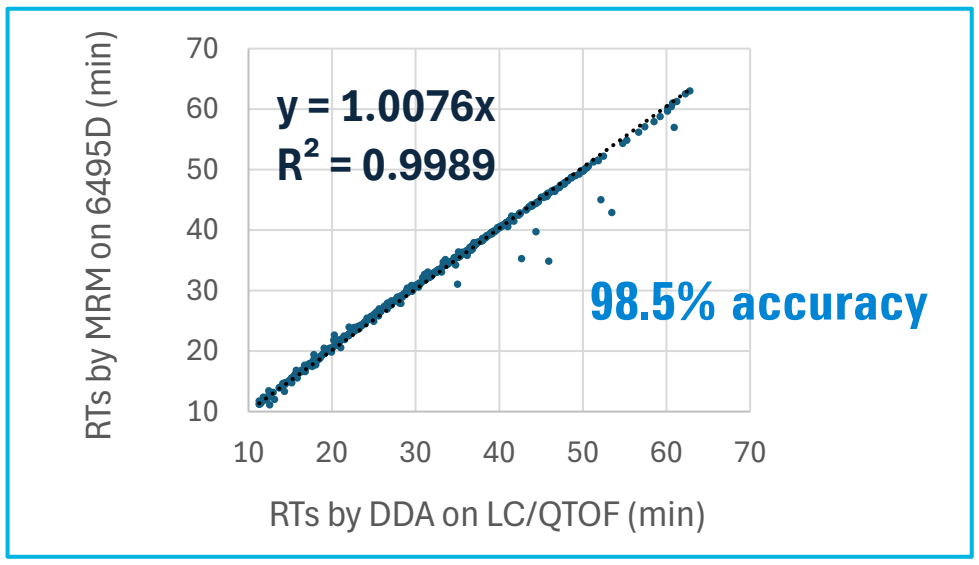

Figure 3. Accuracy of peptide detection by MRM-based method development using Skyline software and 6495D LC/TQ without SIL peptides.

Method Reproducibility

The final large panel targeted proteomic method shows excellent quantification, sensitivity and precision (Table 1 & Figure 4).

Table 1. Summary of the Large panel dMRM method.

Gradient Length (min)	68
LC Flow Rate (nL/min)	200
Total Peptide/Protein Counts	1000/1000
Total MRM Transitions	7942
Median Data Point per Peak	17.13
Median FWHM (min)	0.1
Median Peptide Peak Area CV	7.5%

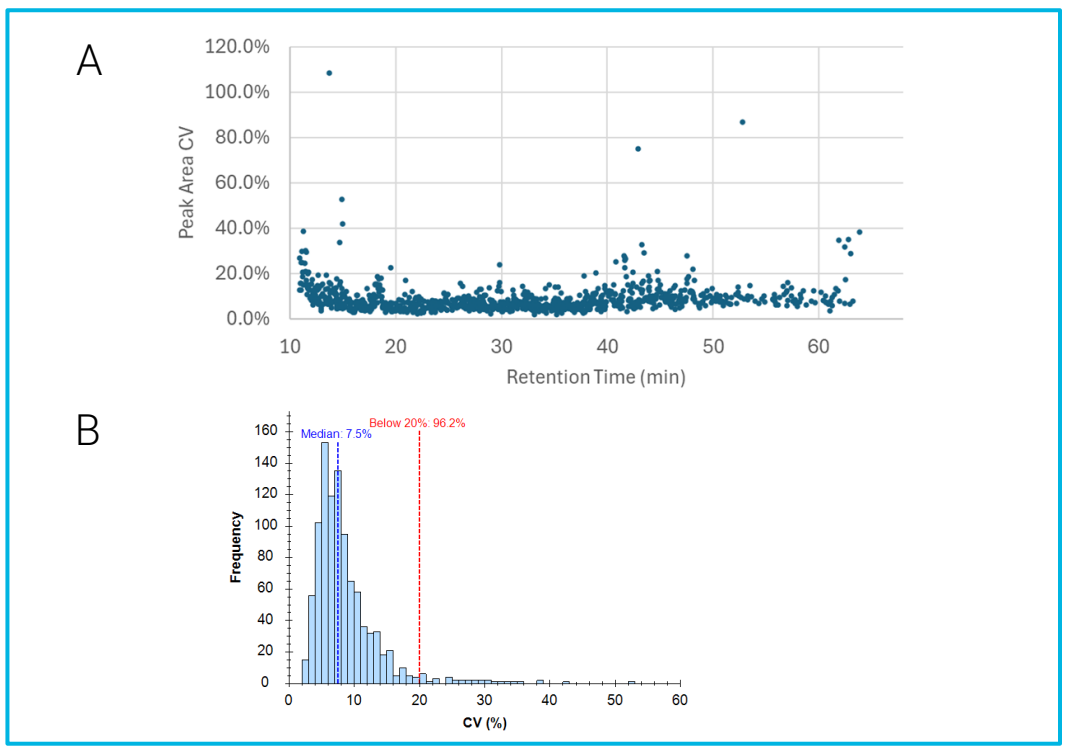

Figure 4. Distribution of peptide peak area CV (%) with 64 ng K562 proteome digest on-column (n=8). A) Plot of peptide peak area CV against LC retention time. B) Histogram of peptide peak area CV distribution.

Results and Discussion

Quantification of Single Cell Proteins

To evaluate system capability for quantifying proteins at single cell level, a sample loading calibration curve ranging from 31.25 pg to 64 ng K562 cell proteome digest on-column was prepared and analyzed in triplicates using the developed 1000-proteins targeted dMRM method (Figure 5 - 7).

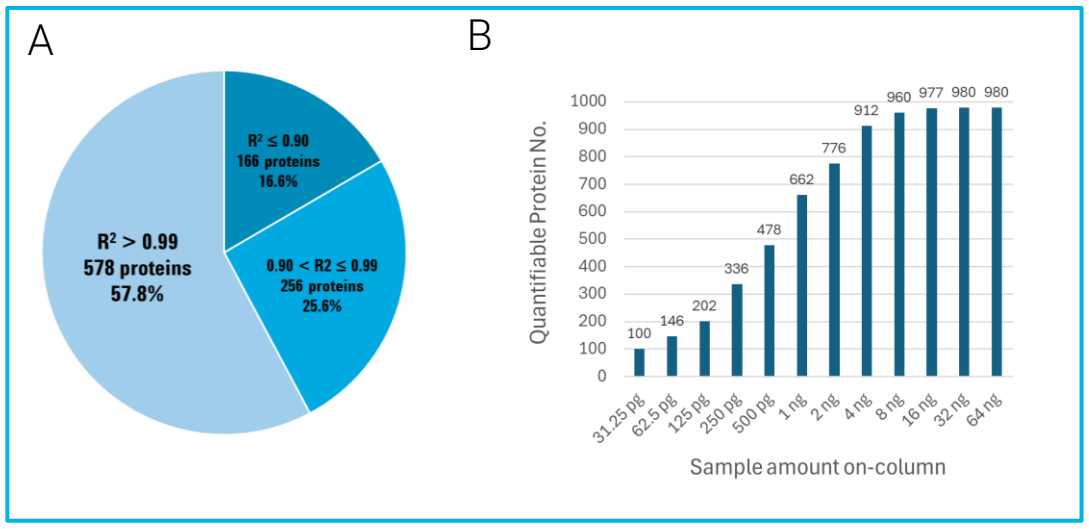

Figure 5. Overview of quantification performance for all the targeted peptides. A) R-squared of linear curve fitting for all the 1000 proteins with sample loading from 31.25 pg to 64 ng on-column. B) Quantifiable protein counts at each sample loading level.

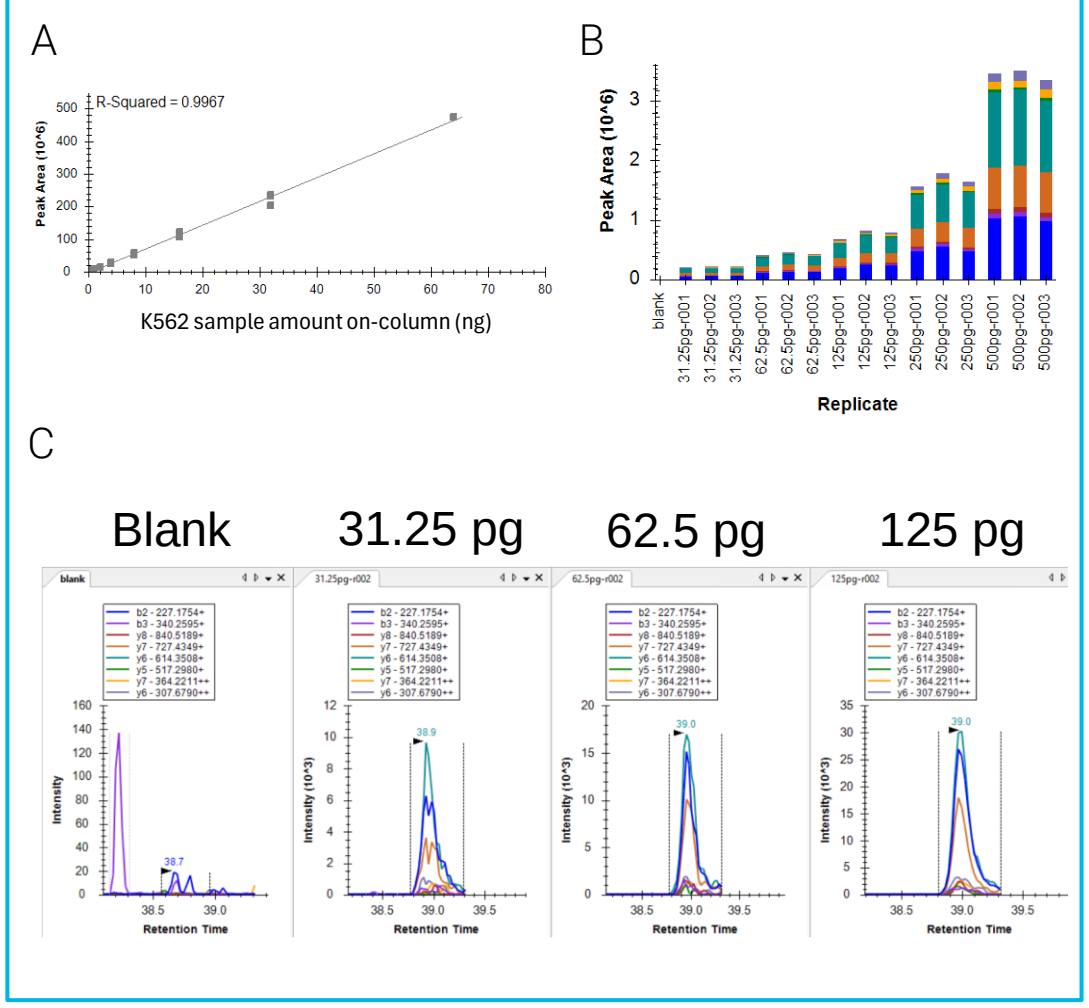

Figure 6. Quantification performance of histone peptide LLLPGELAK. A) Sample loading linear calibration curve. B) Triplicate peptide peak areas at low levels. C) Peptide MRM chromatograms at low levels.

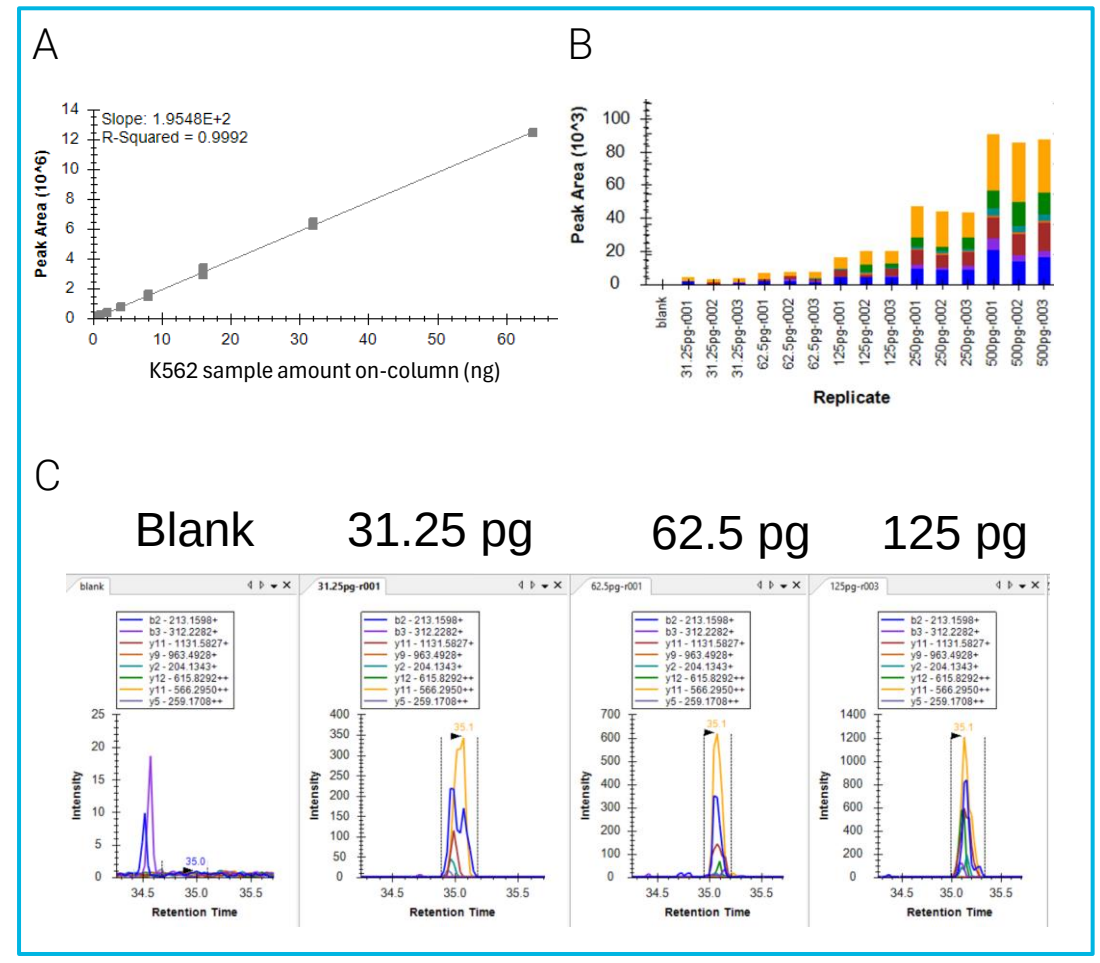

Figure 7. Quantification performance of Poly(rC)-binding protein 1 peptide LVVPATQCGSLIGK. A) Sample loading linear calibration curve. B) Triplicate peptide peak areas at low levels. C) Peptide MRM chromatograms at low levels.

Conclusions

This study demonstrated an enhanced nanoflow LC/TQ system including Evosep One, Newomics ion source and Agilent 6495D LC/TQ for large panel targeted proteome quantification at single cell level:

- The Skyline and 6495D LC/TQ-based dMRM method development workflow is economical and practical as SIL peptides were not required
- The enhanced system shows excellent protein quantification sensitivity, precision and accuracy at single cell level

References

[1]A Rapid and Economical Workflow for Protein Biomarker Discovery Using Agilent 6495D Triple Quadrupole LC/MS System.

Conflict of Interest Statement: All Authors Disclose They Are Employed Either by Agilent Technologies, Inc. or by Newomics, Inc. at the Time of Study Completion.

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